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Emergence of *Aureobasidium pullulans* as human fungal pathogen and molecular assay for future medical diagnosis

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Abstract Despite the great importance of *Aureobasidium pullulans* in biotechnology, the fungus had emerged as an opportunistic human pathogen, especially among immunocompromised patients. Clinical detection of this rare human fungal pathogen presently relies on morphology diagnosis which may be misleading. Thus, a sensitive and accurate quantitative molecular assay for *A. pullulans* remains lacking. In this study, we presented the microscopy observations of *A. pullulans* that reveals the phenotypic plasticity of the fungus. *A. pullulans*-specific primers and molecular beacon probes were designed based on the fungal 18S ribosomal RNA (rRNA) gene. Comparison of two probes with varied quencher chemistry, namely BHQ-1 and Tamra, revealed high amplification efficiency of 104% and 108%, respectively. The optimized quantitative real-time PCR (qPCR) assays could detect and quantify up to 1 pg concentration of *A. pullulans* DNA. Both assays displayed satisfactory performance parameters at fast thermal cycling mode. The molecular assay has great potential as a molecular diagnosis tool for early detection of fungal infection caused by *A. pullulans*, which merits future study in clinical diagnosis.

Introduction

Aureobasidium spp. have been described as yeast-like fungi, which produce yeast-like hyaline conidia and a filamentous growth form (Taylor et al. 2006). This genus was established in 1891 by French scientists Viala and Boyer (Cooke 1959). The well-studied species of this genus, *A. pullulans* is popularly known as the "black yeast" due to its melanin production (Chi et al. 2009). The fungus displays phenotypic plasticity and could be categorized into three distinctive forms, namely smaller elliptical yeast-like cells, large chlamydospores, and elongated branched septate filaments. The colour of its colony progresses from yellow, cream, light pink, or light brown to blackish at a later stage due to chlamydospore production (Chi et al. 2009; Slepecky and Starmer 2009). *A. pullulans* is ubiquitous, and various strains were mainly isolated from soil, plants, wood, damp indoor surface, and indoor air environment (Hawkes et al. 2005; Joshi et al. 2010; Prasongsuk et al. 2005).

As reviewed by Chi et al. (2009), *A. pullulans* is a biotechnologically important yeast. It is well-reported for various applications, such as production of pullulans, extracellular polysaccharide, siderophore, single-cell protein, and hydrolytic enzymes including amylases, proteases, esterases, pectinases, xylanases, and mannanases (Chi et al. 2009; Ravella et al. 2010; Rumbold et al. 2003). Besides that, Manitchotpisit et al. (2011) discovered 21 potential strains of *A. pullulans* which can produce extracellular heavy oil of related structures. Generally, the fungus is regarded as safe for biotechnological and even environmental applications. Therefore, *A. pullulans* has been widely used in organic farming as a biocontrol agent for plant protection, which functions as antagonist in management of indigenous antagonistic potential. A biocontrol product based on this fungus is currently in the market under the trade name Botector® (Schmid et al. 2011).

Despite the great importance of *A. pullulans* in biotechnology, the fungus had emerged as an opportunistic human

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pathogen, especially among immunocompromised patients. *A. pullulans* had been reported to cause nosocomial infection, abscess in the spleen, invasive pulmonary infection, fungemia, peritonitis (among patients on peritoneal dialysis), pneumonia, meningitis, corneal ulcer, catheter-related septicemia, scleral infection, and asthma (Bolignano and Criseo 2003; Clark et al. 1995; Hawkes et al. 2005; Jones and Christensen 1974; Panda et al. 2006; Huang et al. 2008; Salkin et al. 1986; Niedoszytko et al. 2007).

Though regarded as a rare cause of cutaneous infection in human, the increased pathological significance of *A. pullulans* has been reported (Chan et al. 2011; Joshi et al. 2010; Pikazis et al. 2009). Recently, we reported on an *A. pullulans* strain which was co-isolated with *Candida orthopsilosis* from the infected skin of a male patient. This immunocompromised patient has been under several types of medication due to primary aldosteronism, hypertension, and hypertriglyceridemia (Chan et al. 2011). Deep soft tissue mycosis on a 53-year-old woman patient due to *A. pullulans* was reported by Pikazis et al. (2009). In addition, Taylor and co-workers (2006) discovered that respirable-sized conidia of *A. pullulans* were associated with immediate reactivity on skin tests and bound to patient sera IgE, as well as relevant in allergic upper and lower airway diseases. Back in 1997, Redondo-Bellón et al. reported on chromoblastomycosis by *A. pullulans* that caused chronic cutaneous and subcutaneous infection in a 55-year-old man who received a liver transplant. However, a few *A. pullulans* strains were considered as contaminant and to be of low virulence, when isolated from skin scrapping of healthy individuals. According to the 5-year review of 556 dematiaceous hyphomycetes, 75 isolates were *Aureobasidium* spp. Most of these (91%) were unlikely to be pathogenic, whereas the other 9% were considered to be of probable pathogenic significance (Pritchard and Muir 1987).

Furthermore, *Aureobasidium* spp. are difficult to eradicate with standard disinfection procedures, as observed by Wilson et al. (2000) during a pseudo-outbreak investigation on contaminated plastic bronchoscopy stopcocks. As of today, there is no standard treatment for infection caused by *A. pullulans* (Hawkes et al. 2005; Joshi et al. 2010). Amphotericin B alone and a combination with other drugs had been used with variable success (Clark et al. 1995; Hawkes et al. 2005; Huang et al, 2008; Joshi et al. 2010; Pikazis et al. 2009). Today, as never before, certain fungi previously considered as non-pathogenic are commonly spotted in immunocompromised patients (Guarner and Brandt 2011). *A. pullulans* could present a real threat in invasive fungal disease among these patients and could be associated with a high mortality rate. Hence, despite its significant importance in biotechnology, this could present as a major area of concern in clinical treatment as well as in diagnosis.

Clinical diagnosis of rare invasive human fungal pathogens remains a problem that has important treatment implications for immunocompromised patients. Though histopathology continues to be a rapid and cost-effective means of providing a presumptive or definitive diagnosis of invasive fungal infection, the technique is limited by the amount of tissue obtained and architectural distortion of the procedures (Guarner and Brandt 2011). Besides that, clinical diagnosis remains reliant on culture method which is relatively slow, and most of the patients especially in immunocompromised cases have developed severe complications while waiting for the diagnosis results. Pitfalls in these techniques have prompted the use of other techniques, including antigen testing, nucleic acid detection, and radiological imaging (Barnes 2008).

Until most recently, there is no molecular assay reported for clinical diagnosis of *A. pullulans*. Due to limitations in tissue and culture diagnoses, real-time PCR assay has assumed an increased importance in diagnosis of invasive fungal infections. This assay has been applied in detection of important human pathogenic fungal genera, including *Aspergillus*, *Candida*, *Cryptococcus*, *Mucor*, *Penicillium*, *Pichia*, *Microsporum*, *Trichophyton*, and *Scopulariopsis* (Vollmer et al. 2008). However, the three types of diagnostic tools (riboprobe, SCAR primers, and Scorpion-PCR) developed by Schena et al. (2002) were strain-specific and designed for microbial tracking of the particular *A. pullulans* strain L47 used as a biocontrol agent for postharvest diseases. Li et al. (1996) had also reported on hybridization probes designed based on the small-subunit rRNA gene of *A. pullulans*, and these were potentially useful for monitoring and quantifying fungi from the environment. Hence, there is no medical application of real-time PCR assay for diagnosis of *A. pullulans* which has been an increasingly significant rare human fungal pathogen. In this study, we aimed to develop real-time PCR assay for simultaneous detection and quantification of *A. pullulans*. We are reporting on the design of novel oligonucleotides, which were used to develop sensitive quantitative real-time PCR assays for detection of *A. pullulans*. Quantification of pathogen level is critical for rapid diagnosis during infection as it provides valuable information to decide on the treatment regime.

Materials and methods

Cultivation and microscopy

A. pullulans AY4 (Chan et al. 2011) was maintained at 4°C on Sabouraud dextrose agar (SDA) plates or −80°C in

potato dextrose broth (PDB) supplemented with 10% glycerol. The growth of *A. pullulans* AY4 was observed under a stereomicroscope (Leica, Germany). In order to observe morphological changes of *A. pullulans*, the culture was incubated for a longer period (1 to 4 weeks). For microscopic morphology, the fungus was inoculated in concave microscope slides filled with SDA, covered with sterile cover slips and incubated in moist chambers for 3 days at 30°C in the dark. The structure and branching pattern of the immersed hyphae were examined at 10× magnification using a phase-contrast microscope (Leica, Germany).

Genomic DNA isolation

DNA isolation was performed using Wizard Genomic DNA Isolation Kit (Promega, USA) according to the manufacturer's instruction. The isolated genomic DNA was viewed under UV transillumination in 1% agarose, and the DNA concentration was determined using Nanodrop (Thermo Scientific, USA).

Oligonucleotide design for amplification of fungal 18S rDNA

The sequences of PCR primers and probe were selected from the sequences of fungal 18S rRNA genes. The 18S rRNA gene sequence of *A. pullulans* AY4 is available in the GenBank database under accession number HQ215536 (Chan et al. 2011). 18S rDNA sequences from various *A. pullulans* strains were selected from GenBank (http://www.ncbi.nlm.nih.gov/), and alignment of the DNA sequences was performed using BLASTn (Altschul et al. 1997). An online search was performed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to check sequence specificity of the primers and the probe designed (Rozen and Skaletsky 2000). *In silico* specificity check was carried out against human and other fungi sequences available from non-redundant (nr) database. The molecular probe utilized Tamra (tetramethylrhodamine) as quencher. Another probe of the same sequence, which used BHQ1 (black hole quencher 1) as quencher, was selected for comparison. The secondary structure of the molecular probe was observed online using Quikfold (http://dinamelt.bioinfo.rpi.edu/) at the DINAMelt Web Server (Markham and Zuker 2005).

Real-time PCR of fungal 18S rDNA

DNA amplification was carried out in 0.2-mL tubes containing 25 μL reaction mix, and 2 μL of DNA extract was used. The reaction mix consisted of QuantiFast Probe PCR Master Mix (QIAGEN, USA), 400 nM of each 18S rDNA primers, and 200 nM of probe. Fast PCR cycling was performed on the CFX96 system (Bio-Rad Laboratories, USA) with preliminary denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing-extension at 60°C for 30 s, with a fluorescence acquisition step at the end of the annealing-extension step. The setting and amplification data were managed using CFX Manager Version 1.6 (Bio-Rad Laboratories, USA). To validate the amplification sensitivity of the 18S rDNA assay, serial dilution of fungal DNA concentrations (10 ng to 10 fg) was tested. The detection limit of low target concentrations was evaluated on the assay sensitivity. Agarose gel (1.5%) electrophoresis was performed to view the amplified PCR product. The expected size of the amplified band was 368 bp.

Statistical analyses

Data analysis was done using Excel 2007. For each quencher, the slope, R^2, and y-intercept values of the regression curves were obtained, and both sets of real-time PCR data generated from quenchers of different chemistry were compared by a one-way ANOVA test with $P<0.05$ (La Paz et al. 2007).

Results

Fungal morphology

In Fig. 1, the colony morphology of *A. pullulans* AY4 grown on SDA varied depending on culture age. The most significant changes that could be observed on its morphological properties were the changes in its colour. *A. pullulans* grown on SDA appeared to be pinkish after 3 days of incubation at 30°C (Fig. 1a). However, after 1-week incubation, the colony started to turn brownish (Fig. 1b). After incubation for almost 3 weeks, the colony was observed to be dark brown in colour (Fig. 1c), and the colour continued to become darker after 4 weeks (Fig. 1d). Based on the colony formed on SDA agar, *A. pullulans* was also observed to have white aerial mycelium (branching-like structure) that formed at the edge of the colony. Figure 2 shows the blastospores produced and melanized hyphae with intercalar synchronous conidiogenesis of *A. pullulans* after inoculation and incubation in SDA for 3 days.

Oligonucleotide design

Specific primer pairs and molecular beacon probe were designed using Primer-BLAST and screened against GenBank non-redundant (nr) database for additional homology

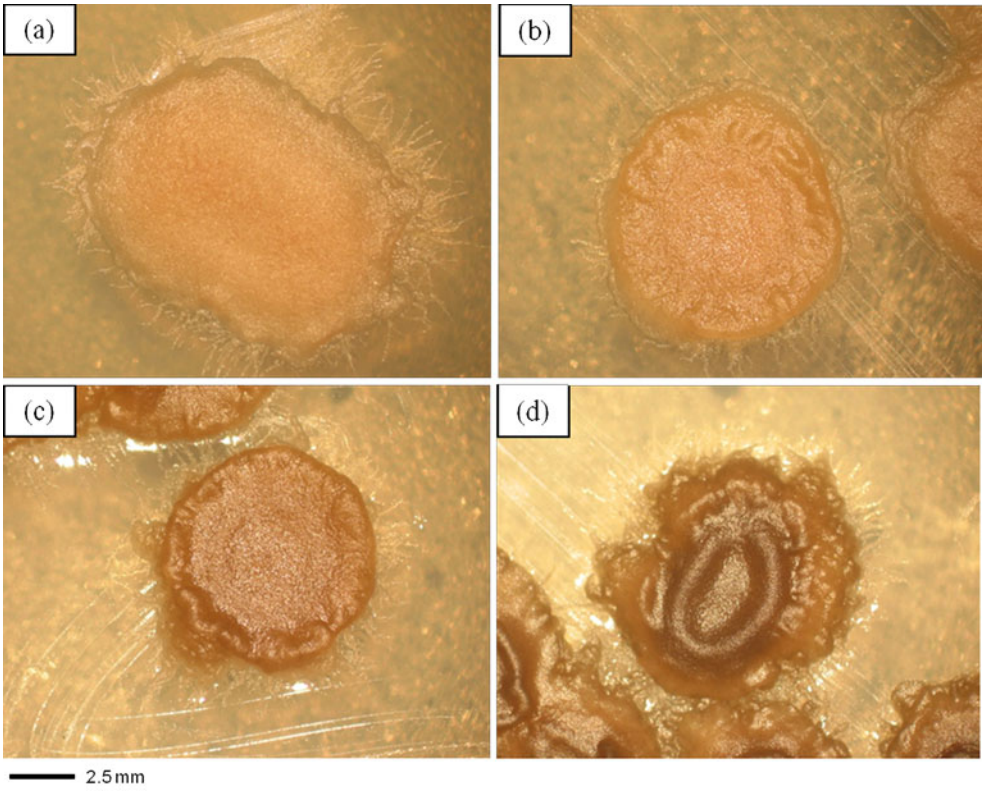

Fig. 1 Colony morphology of *A. pullulans* at 5× magnification under stereomicroscope after **a** 3 days, **b** 1 week, **c** 3 weeks, and **d** 4 weeks

against human and other fungal sequences, including those fungal sequences from related fungi shown in Fig. 3. Sequences that showed no homology with human and fungal sequences were selected. After the homology analysis, the primer sequence 5′-TAC GGT GAA GCT GCG TGA TGG CT-3′ was selected as forward primer and named as AP19F. The primer sequence 5′-TGG GTA ATT TGC GCG CCT GCT-3′ was selected as reverse primer and named as AP386F. The sequence 5′(6Fam)-ACC CCA ACT TCG GAA GGG GT-(Tamra)3′ was selected as molecular beacon probe and named as APFTam1. Another probe of the same sequence, utilizing BHQ1 as the quencher, was used for comparison and named as APFaQ1-2. The positions of primers and probe are shown in Fig. 4, and multiple sequence alignment of partial 18S rDNA sequences of 10 strains of *A. pullulans* showed conservation among the geographically diverse strains. These results also indicate that 18S rRNA gene could be used to design species-specific probe. The secondary structure of molecular beacon probe is also shown in Fig. 4.

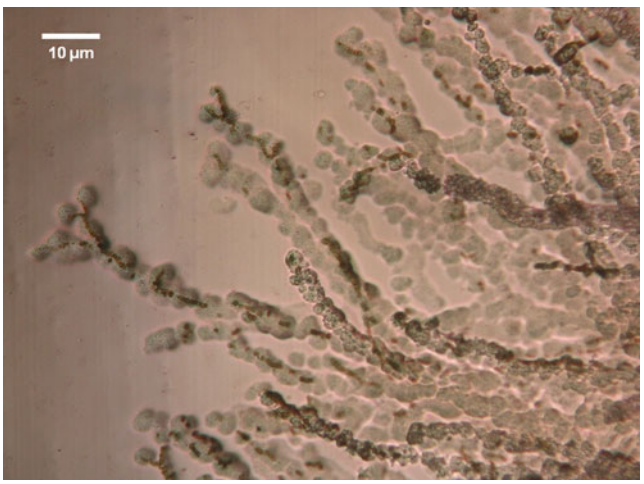

Fig. 2 Production of blastospores and melanized hyphae with intercalar synchronous conidiogenesis of *A. pullulans* after inoculation in SDA for 3 days. This was observed at 10× magnification using a phase-contrast microscope

Real-time PCR of fungal 18S rDNA

Real-time PCR assay of *A. pullulans* was successfully optimized, and the amplification results are shown in Figs. 5 and 6. Molecular beacon probe of APFTam-1, which uses 6-Fam as fluorophore and Tamra as quencher, generates a good result. In Fig. 5a, distinct fluorescence signals were generated by APFTam-1 probe for up to 1-pg concentration of *A. pullulans* AY4 DNA. The real-time PCR assay of *A. pullulans* AY4 is specific as an amplified single band of 368 bp shown in agarose gel in Fig. 5b. Figure 5c shows the relation between the threshold cycle and the apparent amount of *A. pullulans* DNA with high linearity of R^2= 0.9983. The assay allows accurate quantification of *A. pullulans* DNA over the range of 1 pg to 10 ng with a PCR efficiency of 104%. APFaQ1-2 probe, which uses 6-Fam as

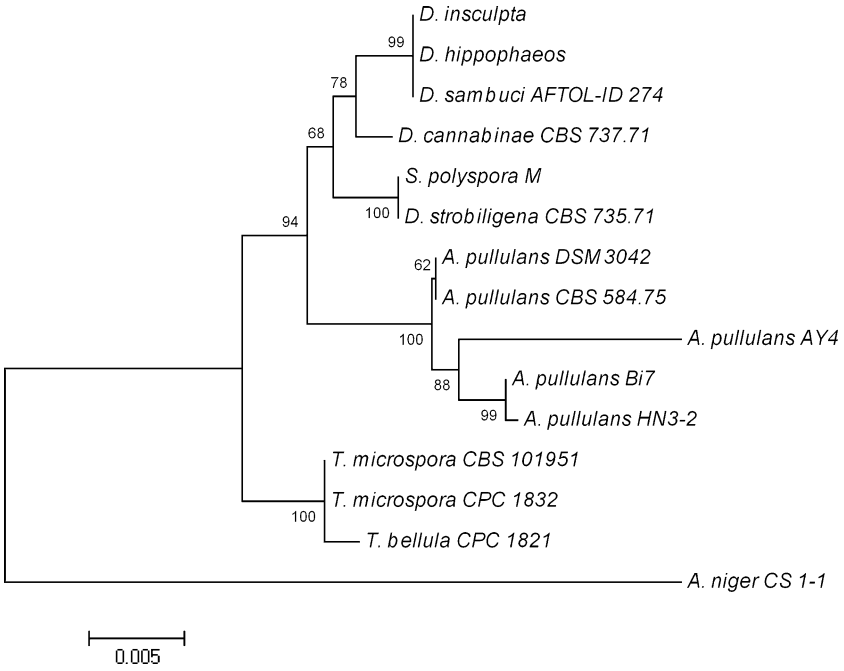

Fig. 3 Neighbor-joining tree based on 18S rRNA gene sequences showing the relationship between fungi from different genus and *A. pullulans*. Organisms used are *Dothidea insculpta* (U42474), *Dothidea hippophaeos* (U42475), *Dothidea sambuci* (NG012432), *Dothiora cannabinae* (DQ479933), *Sydowia polyspora* (AY150054), *Delphinella strobiligena* (AY016341), *A. pullulans* (EU707932, DQ471004, HQ215536, EU707929, DQ486709), *Teratosphaeria microspora* (GU214520, EU167572), *T. bellula* (GU214601), and *Aspergillus niger* (HM590646). Numbers in parentheses indicate the GenBank accession numbers

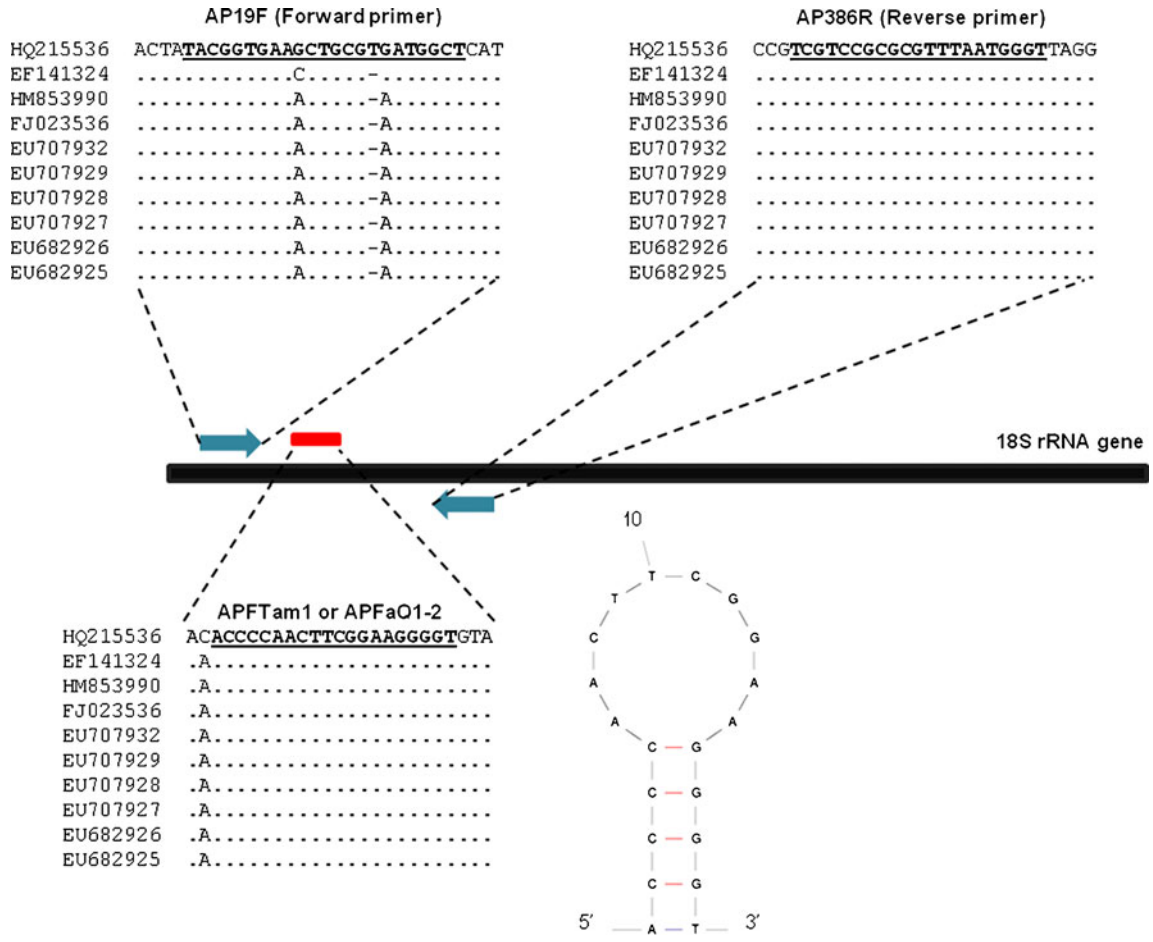

Fig. 4 Schematic diagram showing designed oligonucleotides from18S rRNA gene of *A. pullulans*. Multiple alignments with other sequences of *A. pullulans* strains (indicated by the GenBank accession numbers) are presented. Secondary structure of molecular probe used in real-time PCR assay of *A. pullulans* is indicated

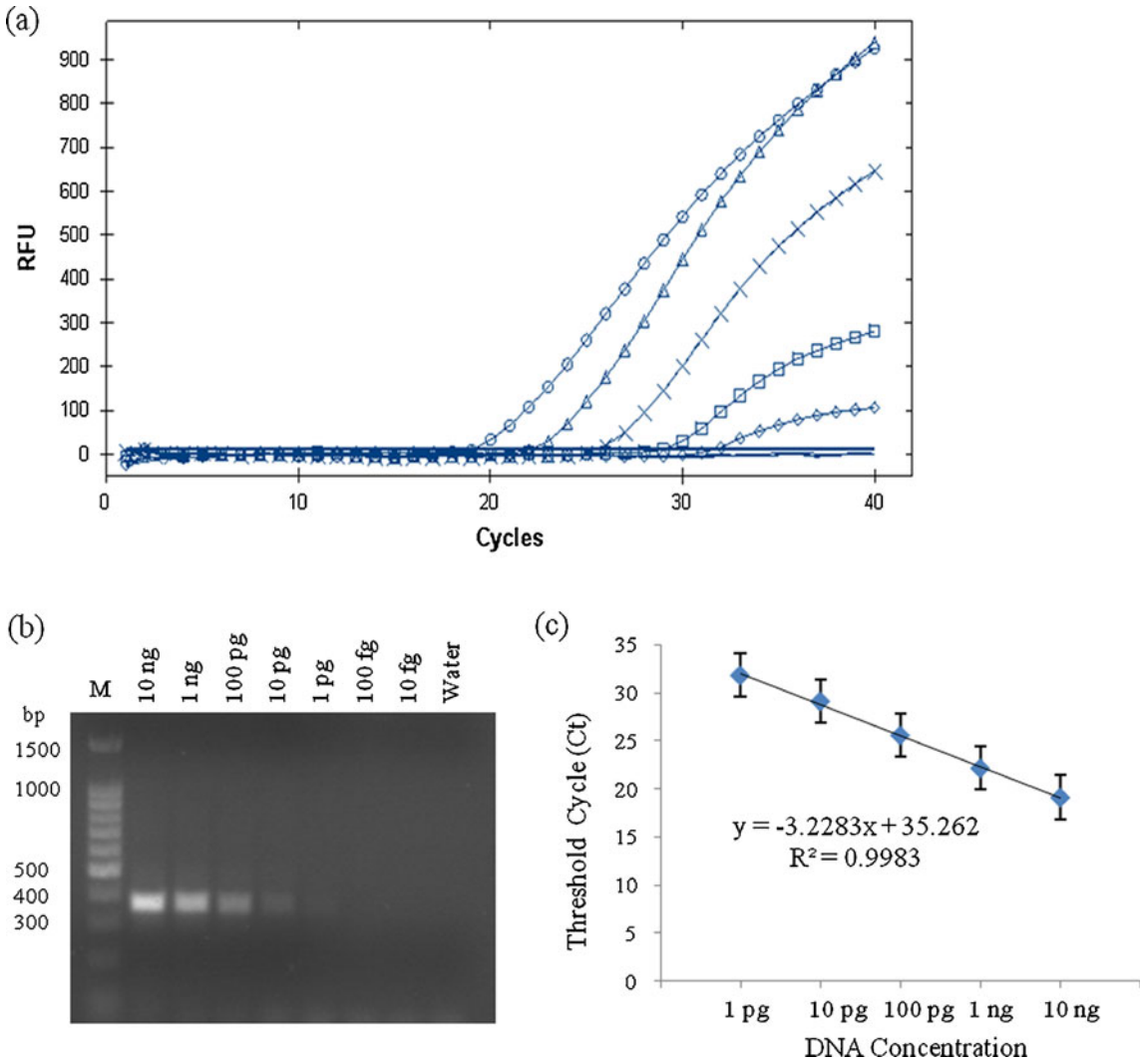

Fig. 5 **a** Sensitivity of real-time PCR assay using a series of 10-fold dilutions of genomic DNA from *A. pullulans* AY4. The DNA concentrations used which showed distinct fluorescence signals were 10 ng (○), 1 ng (Δ), 100 pg (×), 10 pg (□), and 1 pg (◊). Distinct fluorescence signals were generated by APFTam1 probe for up to 1-pg concentration of *A. pullulans* AY4 DNA. *RFU* relative fluorescence unit. **b** Verification of the amplicon generated from PCR assays. M: 100 bp DNA Ladder (Promega). **c** Relation between the threshold cycle and the apparent amount of *A. pullulans* AY4 DNA using real-time PCR assay. The PCR amplification efficiency is 104%

fluorophore and BHQ1 as quencher, generated a better result. As shown in Fig. 6a, the distinct fluorescence signals were generated by APFaQ1-2 probe for up to 100-fg concentration of *A. pullulans* AY4 DNA. The use of BHQ1 as quencher resulted in a more sensitive amplification. The specificity of bands amplified (Fig. 6b) was comparable to the bands generated using APFTam-1 probe. Figure 6c shows the relation between the threshold cycle and the apparent amount of *A. pullulans* DNA with high linearity of R^2=0.9942. The assay allowed accurate quantification of *A. pullulans* DNA over the range of 1 pg to 10 ng with a PCR efficiency of 108%. The fast PCR cycling had allowed these assays to complete within 1 hour and 15 min.

A one-way ANOVA test with $P<0.05$ was used for comparison of fluorescence signals generated by APFTam-1 and APFaQ1-2 probes. The null hypothesis of this comparison was that there was no difference in the fluorescence signals generated by APFTam-1 and APFaQ1-2 probes, whereas the alternative hypothesis was that there was a difference in the fluorescence signals generated. The value of F was 0.047539, which was less than the critical value of F at the 5% level of significance, which was 5.317655. The P value was 0.832861, which was higher than 5% level of significance ($P>0.05$). This showed that there was no significant difference in the fluorescence signals generated by APFTam-1 and APFaQ1-2 probes. Therefore, both of these probes could be used for real-

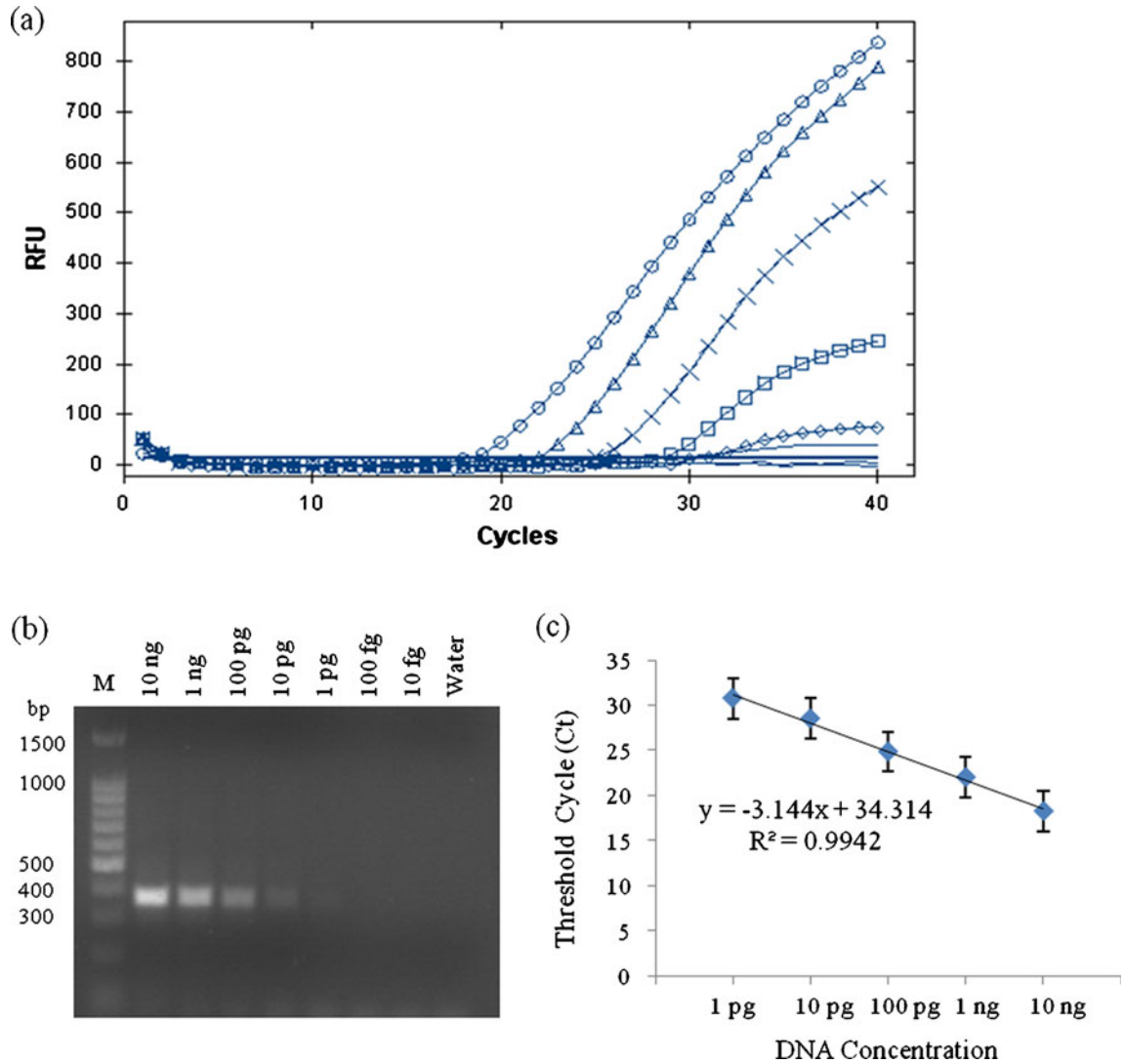

Fig. 6 **a** Sensitivity of real-time PCR assay using a series of 10-fold dilutions of genomic DNA from *A. pullulans* AY4. The DNA concentrations used which showed distinct fluorescence signals were 10 ng (○), 1 ng (Δ), 100 pg (×), 10 pg (□), 1 pg (◊) and 100 fg (−). Distinct fluorescence signals were generated by APFaQ1-2 probe for up to 100-fg concentration of *A. pullulans* AY4 DNA. *RFU* relative fluorescence unit. **b** Verification of the amplicon generated from PCR assays. M: 100 bp DNA Ladder (Promega). **c** Relation between the threshold cycle and the apparent amount of *A. pullulans* AY4 DNA using real-time PCR assay. The PCR amplification efficiency is 108%

time PCR quantification of *A. pullulans* without a significant difference.

Discussion

To the best of our knowledge, this is the first report on molecular beacon real-time quantitative PCR assay for detection of the rare human pathogen *A. pullulans* based on the small subunit of ribosomal gene. 18S rDNA is repetitive in nature of over 100 copies per fungal genome, which makes this gene a good target and the rDNA-based amplification easier (Wu et al. 2003). In this study, the probe was intended to be designed as TaqMan probe. However, it also formed secondary structure as molecular beacon. Molecular beacon probe is single-stranded probe with a hairpin-shaped structure and complementary ends (stem) that maintain the close proximity of fluorophore and quencher. During amplification, molecular beacon hybridizes to the target sequence and unfolds, thus allowing the fluorescent emission (La Paz et al. 2007). This kind of hairpin-shaped structure could relatively improve the specificity and prevent mismatch binding. Generally, different chemistry of fluorophores and quenchers was developed to improve specificity and sensitivity of real-time PCR assay as well as to decrease cost. In our case, BHQ1 is generally more cost-effective than Tamra, as well as resulting in a more sensitive amplification. High

sensitivity in quantitative real-time PCR assay offers advantage over traditional culture method, histopathology diagnosis, and even conventional PCR, as diagnosis could be carried out rapidly and less samples from patient is required.

In clinical diagnosis, morphology characterization is generally performed on patient samples. From the cultures or tissue samples, the fungal pathogens are identified based on the morphology and characteristics of spores. Yet, this technique is labor intensive and very dependent on the skills of experienced medical laboratory technicians. Complications in microscopic analysis are also derived from mixed infection and/or contaminated culture. For instance, in the case presented by Hawkes et al. (2005) of *A. pullulans* infection in an infant, the patient deteriorated clinically with cardiac arrest 3 days before the first positive culture, coupled with bacterial isolation of *Pantoea agglomerans* from the blood culture, which complicated the interpretation and observation of clinical manifestation (Hawkes et al. 2005). In addition, molecular identification and diagnosis is an important approach towards accurate detection as phenotypic plasticity of *A. pullulans* is generally observed from morphology characterization. Based on Zalar et al. (2008), the colour changes in colony morphology of *A. pullulans* are due to the production of melanized hypae and conidia. Its colony form varies depending on the type of single carbon substrates (sugars and sugar alcohols) used in the growth medium, colony age, incubation temperature, light cycle, and substrate type (Slepecky and Starmer 2009). Bolignano and Criseo (2003) had also reported on the use of fungal cultures to diagnose the presence of *A. pullulans* causing nosocomial infection. The appearance of the fungus rapidly changed within the first week of incubation (Bolignano and Criseo 2003). In the case presented by Joshi et al. (2010), yeast-like colonies suggestive of *Candida* species were isolated from blood culture of an 11-year-old boy after allogeneic stem cell transplant. However, on subculture, the colony characteristic matured and was suggestive of *A. pullulans* fungemia (Joshi et al. 2010). Therefore, in order to address the need for early, sensitive, and accurate diagnosis, molecular assay of *A. pullulans* based on quantitative real-time PCR could be a well-sought alternative.

In conclusion, quantitative real-time PCR assay for detection of *A. pullulans* using novel molecular probes was successfully developed, and its potential merits future study. Our preliminary finding showed that this assay fulfills the demand for a sensitive, specific, and cost-effective diagnostic method. The PCR assay should be validated with clinical samples to evaluate its sensitivity and specificity over a larger sample population prior to development as diagnostic kit.

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